Internalization of Sapovirus, a Surrogate for Norovirus, in Romaine Lettuce and the Effect of Lettuce Latex on Virus Infectivity

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Noroviruses are the leading cause of food-borne outbreaks, including those that involve lettuce. The culturable porcine sapovirus (SaV) was used as a norovirus surrogate to study the persistence and the potential transfer of the virus from roots to leaves and from outer to inner leaves of lettuce plants. Treatment of lettuce with SaV was done through the roots of young plants, the soil, or the outer leaves of mature plants. Sampling of roots, xylem sap, and inner and outer leaves followed by RNA extraction and SaV-specific real-time reverse transcription (RT)-PCR was performed at 2 h and on postinoculation days (PID) 2, 5, 7, 14, and/or 28. When SaV was inoculated through the roots, viral RNA persisted on the roots and in the leaves until PID 28. When the virus was inoculated through the soil, viral RNA was detected on the roots and in the xylem sap until PID 14; viral RNA was detected in the leaves only until PID 2. No infectious virus was detected inside the leaves for either treatment. When SaV was inoculated through the outer leaves, viral RNA persisted on the leaves until PID 14; however, the virus did not transfer to inner leaves. Infectious viral particles on leaves were detected only at 2 h postinoculation. The milky sap (latex) of leaves, but not the roots’ xylem sap, significantly decreased virus infectivity when tested in vitro. Collectively, our results showed the transfer of SaV from roots to leaves through the xylem system and the capacity of the sap of lettuce leaves to decrease virus infectivity in leaves.

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uman noroviruses (NoVs) are the leading cause of food-borne illnesses, accounting annually for 58% of cases (47). They cause 26% and 11% of food-borne-associated hospitalizations and deaths in the United States, respectively (47). An association between food-borne illnesses and the consumption of fresh fruits and vegetables is increasingly being recognized (5). Noroviruses account for an increasing number of food-borne illnesses associated with fruits and vegetables (16, 23, 25, 35, 37). Because leafy greens are consumed with minimal processing and NoVs are environmentally stable and have a low infectious dose (~18 to 1,000 viral particles) (20), NoV-contaminated uncooked vegetables and salads constitute a serious risk to consumers. Therefore, preventive efforts should focus on reducing the initial preharvest contact between these viruses and vegetables as well as postharvest contamination.

While the majority of reported NoV outbreaks have been due to the contamination of produce handled by infected workers (5), on-the-farm sources of contamination, such as application of contaminated organic amendments and irrigation water, can also lead to preharvest produce contamination (53). Of great concern are internalized pathogens since they are protected from postharvest surface washing and sanitization procedures (55). Plant bacterial pathogens can access the plant’s interior either by penetrating the roots’ surface or by entering the leaves through stomata or damaged regions (11, 66). However, previous studies have led to conflicting results regarding the internalization of human bacterial pathogens through the roots of vegetables (14, 39, 56, 67), while limited entry to the leaf internal tissues has been shown to occur through the stomata (46, 51). Whether NoVs remain on the leaf surface or internalize via stomata or roots is largely unknown. Because NoVs have a very small size (28 to 35 nm) compared to that of bacteria, they may be able to internalize passively through the roots during plant water absorption or through the stomatal openings during leaf transpiration. To date, however, only two studies have assessed the potential internalization of enteric viruses through the root system (55, 61) but not the stomata, although it has been shown that these viruses preferentially attach to stomata (15, 62). Urbanucci et al. (55) have found that canine calicivirus (CaCV), a Vesivirus used as an NoV surrogate, internalized with various frequencies in lettuce plant seedlings (5 days of age) grown hydroponically over a period of 9 days. In a more recent study, investigators found that murine NoV (MNV) inoculated through the roots into hydroponically grown young lettuce plants (20 days of age) internalized through postinoculation day (PID) 5 with higher titers than when the plants were grown in soil (61). Both studies used young plants that might not be representative of mature lettuce plants at harvest, and neither assessed the persistence of the viruses inside the plant until harvest time. Therefore, more studies are needed to determine whether food-borne viruses internalize through the roots and/or stomata and to assess the long-term persistence of these viruses in leafy greens.

Despite the increased importance of human NoVs as food-borne pathogens, the inability to grow the virus in vitro has hindered research on virus transmission and its control in vegetables. However, surrogate culturable viruses, such as feline calicivirus (FCV), MNV, and a cell culture-adapted enteric calicivirus, porcine sapoviruses (SaV; Cowden strain), are currently being used (63). Sapoviruses are directly relevant to human health since recent studies suggested that human SaV-associated gastroenteri-
tis is becoming more prevalent worldwide (26, 41, 64). In addition, SaVs have been detected in environmental samples such as wastewater, contaminated river water, and clams and oysters (13, 26), suggesting that SaVs, like NoVs, are transmitted via the fecal-oral route through contaminated foods and water (65). The Centers for Disease Control and Prevention also listed SaVs among the viral pathogens causing food-borne illnesses in the United States (47). Also, certain porcine SaVs and NoVs are genetically and/or antigenically closely related to the human strains, suggesting the potential for zoonotic infections (40, 58, 59). Besides the porcine SaV Cowden strain being similar in size (28 to 35 nm) to NoV and having a surface charge range similar to that of NoV (isoelectric points of 5.36 and 5.9 to 6.9, respectively) (21, 22), it has been shown recently that porcine SaV shares characteristics similar to those of human NoV, including resistance to low and high pH (42, 57), heat (56°C), and chlorine treatments (57). However, unlike human NoV, porcine SaV is culturable, allowing quantification of infectious virus. Therefore, porcine SaV was chosen as a suitable surrogate for assessing human NoV contamination in lettuce. We used lettuce as a model salad crop because it is consumed in large quantities in the United States with minimal processing and it has been implicated in a number of NoV food-borne outbreaks (5, 16, 37). The objective of our study was to assess the persistence and the potential transfer of porcine SaV, used as a surrogate for human NoVs or SaVs, from roots to leaves and from outer to inner leaves of lettuce plants. For this purpose, lettuce plants at age 4 or 8 weeks were contaminated (inoculated) either through the roots, the soil, or the outer leaves, and the virus or viral RNA was tracked in the roots, the leaves, xylem sap, and inner and outer leaves.

**MATERIALS AND METHODS**

**Propagation of SaV.** The cell culture-adapted porcine SaV Cowden strain was propagated in the porcine kidney cell line (LLC-PK) (ATCC CL-101) as described previously (10, 19). Briefly, LLC-PK cells were cultured at a density of 3 × 10^5 cells/flask (175 cm^2) and incubated for 3 days at 37°C. After the cells were washed, SaV was inoculated at a multiplicity of infection (MOI) of 0.1 and incubated for 1 h at 37°C. The culture medium containing the virus was minimal essential medium (MEM) containing 1% nonessential amino acids and 1% antibiotic-antimycotic cocktail (Invitrogen, Carlsbad, CA), supplemented with 50 μM of bicine (glycochenodexyoscholic acid) (Sigma-Aldrich, St. Louis, MO). The cells were incubated for an additional 3 to 5 days at 37°C. SaVs were released by applying three cycles of freeze-thawing. The virus was separated from cell debris by centrifugation at 2,500 × g for 20 min. The supernatant containing SaVs was aliquoted, stored at −20°C, and used in all subsequent experiments. Virus titers were assayed using real-time quantitative reverse transcription-PCR (qRT-PCR) and infectivity assays (as described below). The qRT-PCR titer of the SaV stock was determined using a standard curve generated from serial dilutions of a DNA plasmid containing a 3-kb sequence of the SaV genome as described earlier (57). The infectious titer of the SaV stock was 10^6 50% tissue culture infective dose (TCID_{50})/ml corresponding to a qRT-PCR titer of 10^{10} genomic equivalent (GE)/ml.

**Inoculation of lettuce plant with SaV.** Seeds of romaine lettuce cultivar Tall Guizanne Elite (Siegers Seed Co., Holland, MI) were grown in 200-cell trays containing Fafard superfine germinating mix (Conrad Fafard, Agawam, MA) under greenhouse conditions as described previously (1). At 4 weeks of age, seedlings were transferred to 15-cm-diameter pots containing sterile soil (Wooster sandy loam) and were fertilized biweekly using Osmocote slow-release fertilizer. Three different scenarios were evaluated to assess SaV mode of transmission in lettuce plants.

(i) **SaV inoculation through the roots.** To study the potential transmission of SaV from contaminated roots to leaves, lettuce seedlings at 4 weeks of age (~4- to 5-leaflet stage) were used, and the soil was removed from the roots by gentle washing with sterile water. Roots were submerged in a 2-ml SaV stock solution for 2 h at room temperature. Seedling leaves were protected from cross-contamination from the SaV inoculum using a Parafilm wrap. The roots of all treated seedlings along with mock-treated (MEM buffer) control seedlings were washed three times in sterile water, transferred to new 15-cm-diameter pots containing sterile soil (Wooster sandy loam), and placed inside biosecurity safety level 2 plant growth chambers under controlled conditions (12-h photo period; 20°C daytime/15°C nighttime temperatures; 60% relative humidity). The pots were placed inside plastic trays. Water was added daily to the trays and thus was delivered to the plants through capillary force without direct contact with the leaves. Sampling of leaves and roots was performed at 2 h postinoculation (defined as PID 0) and on PID 2, 5, 7, 14, and 28. Samples were processed as described below.

(ii) **SaV inoculation through the soil.** To study the potential transmission of SaV from soil to roots and then to leaves, SaV was inoculated through the soil. Lettuce plants were used at 8 weeks of age, just 2 weeks prior to being market ready. Lettuce leaves were protected by inserting the head inside an inverted sterile Whirl-Pak bag (Nasco, Salida, CA). The bottom edge of the bag was cut to allow for air to pass from the top of lettuce heads while protecting the leaves from soil contact. Five milliliters of SaV stock solution were suspended in 25 ml of sterile water and applied carefully over the soil surface without leaf or stem contact. Treated and nontreated control plants were placed inside growth chambers under the same conditions as described earlier. Watering was performed as described earlier. Sampling of leaves and roots was performed at PID 0, 2, 5, 7, and 14. Xylem sap oozing from the cut stems at the root side were collected over a 1- to 2-h period (total volume of ~150 to 200 μl) as described previously (52). The roots were washed several times in sterile water to completely remove soil particles. All samples were processed as described below.

(iii) **SaV inoculation through the outer leaves.** To study the potential transmission of SaV from outer leaves to inner leaves, outer leaves were inoculated with SaV, while inner leaves were protected. Specifically, approximately 6 to 7 inner leaves of mature lettuce heads (at 8 weeks of age) were covered using an inverted sterile open-end Whirl-Pak bag (as described earlier), and the outer leaves (~6 to 7 per plant) were spot inoculated with a total of 1 ml SaV stock solution. Inoculated leaves were marked with an adhesive round label (1 cm in diameter) (Diversified Biotech). Leaves were allowed to dry in a biosafety hood before transferring all the plants to growth chambers. Watering was performed as described earlier. Samplings were performed at PID 0, 2, 5, 7, and 14, whereby three inner and three outer leaves per plant from treated and control plants were separately pooled and processed as described below.

**Processing of lettuce samples.** Samples (leaves and roots) were weighed, cut into small pieces, suspended in elution buffer containing MEM supplemented with 2% fetal bovine serum (18), and homogenized on ice using a polytron homogenizer (Cole-Parmer Instruments) at maximum speed for 1 min. Specifically, for lettuce plants age 4 to 5 weeks, all the leaves and roots were harvested (weight of <3 g; volume of elution buffer used, 10 to 12 ml). For lettuce plants age 6 to 10 weeks, all roots as well as 10 random leaves per plant were randomly selected and cut in half along the midrib, and only one half per plant was processed. Leaf halves were cut into small pieces, mixed, and pooled into one sample (weight of 10 to 12 g; volume of elution buffer used, 35 to 40 ml). A concentration step was used based on the method of Rutjes et al. (45) with minor modifications. The samples were centrifuged twice at 3,724 × g for 10 min to remove plant debris. The supernatants were ultracentrifuged at 112,700 × g for 1.5 h to concentrate the virus. The pellets were suspended in 500 μl of 0.01 M phosphate-buffered saline (PBS; pH 7.4). Half of this volume was used for RNA extraction, and depending on the results of qRT-PCR, the other half was used for infectivity assays (as described below).

**RNA extraction.** Viral RNA was extracted from 250 μl of the processed leaf and root samples and 100 μl of xylem sap using the RNeasy
minikit (Qiagen, Valencia, CA). A sterile water sample was extracted with every run to serve as an RNA extraction control in addition to experimental lettuce (roots and leaves) control samples. The RNA was eluted in 50 μl nuclelease-free water and stored at −20°C. RNA samples within the same treatment were analyzed together for determining the qRT-PCR titer.

**Real-time RT-PCR for the detection of SaV.** One-step TaqMan SaV-specific qRT-PCR (57) was used to estimate the virus concentration in lettuce leaf and root samples. Briefly, 2 μl of each sample was mixed with 18 μl of master mix prepared using the Qiagen one-step RT-PCR kit (Qiagen). The master mix contained 1 × PCR buffer, 400 μM deoxy-nucleoside triphosphates (dNTPs), 200 nM each primer, 100 nM TaqMan probe, 0.8 μl of enzyme mix, and 8 units of RNAses (Promega, Madison, WI). The amplification cycle consisted of the reverse transcription step (50°C for 30 min), one cycle at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 57.5°C for 1 min. qRT-PCR was run in duplicate for each sample. Negative (nuclease-free water) and positive (SaV of known threshold cycle [C_{T}] value) samples were included with each qRT-PCR run. qRT-PCR was performed with a Mastercycler realplex (Eppendorf, Germany). An internal RNA control was spiked into randomly selected negative samples to check for RT-PCR inhibitors as described previously (57). None of the lettuce RNA samples showed any PCR inhibitors.

To generate a standard curve and determine the detection limit of the qRT-PCR and infectivity assays, lettuce plants were bought from the local market, and the leaves were spot inoculated in triplicate with 1 ml of serially diluted SaV (10^6 to 10^0 TCID_{50}/ml). Leaves used as a control were mock inoculated with MEM buffer. The spots were allowed to dry in the biosafety hood for about 2 h, and then the leaves were processed as described above to elute and concentrate the virus. The concentrated virus was suspended in 500 μl of phosphate-buffered saline (PBS), and half of the volume was used to extract RNA followed by SaV-specific qRT-PCR, and the other half was tested by infectivity assay as described below. The experiment was performed three times with triplicate leaf samples per diluted treatment. SaV RNA titer in lettuce was calculated using the equation \( y = -0.2914x + 12.851 \quad (R^2 = 0.99) \), where \( y \) is the SaV log qRT-PCR titer (GE/reaction) and \( x \) is the \( C_T \) value. The titer was then adjusted to GE/g of lettuce tissue.

**SaV infectivity assay.** The infectivity titer of SaV eluted from the lettuce samples was estimated in LLC-PK cells using immunohistochemical staining as described previously (57). In short, LLC-PK cell monolayers in 96-well plates were infected in quadruplicate with serially diluted (1:10) samples and incubated for 72 h at 37°C. The cells were fixed for 30 min at room temperature using 3% formalin in PBS and were permeabilized for 5 min using 1% Triton X-100 in PBS. The primary antibody used was generated in our laboratory following hyperimmunization of guinea pigs with virus-like particles of porcine SaV (24). The secondary antibody was used for statistical analyses. One-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was used to determine significant differences between different time points within each treatment. Each greenhouse experiment was performed three times with triplicate samples per time point per treatment. Differences in means were considered significant when the \( P \) value was < 0.05. Data were expressed as means ± standard errors (SE).

**RESULTS**

Infecitivity detection limit of SaV recovered from lettuce. The minimum infectious amount of SaV that was detected by our infectivity assay was 1.44 ± 0.14 log_{10} TCID_{50}/g. This titer corresponded to a qRT-PCR titer of 5.23 ± 0.27 log_{10} GE/g. Only samples with a qRT-PCR titer above this detection limit were tested for infectious viruses. On average, the infectivity titer of the recovered virus was 1.23 ± 0.12 log_{10} GE/g less than that of the original SaV inoculum. This difference in recovered viral infectivity titer when the virus was inoculated onto lettuce leaves could be due to virus loss during the elution step and/or to various inactivating/damaging factors released from lettuce during the homogeniza-
The SaV RNA persisted in the roots of lettuce plants until PID 28 (Table 1). However, the RNA titer decreased significantly by 3.76 log10 GE/g on PID 2 and then was maintained at similar levels through PID 14. By PID 28, the viral RNA decreased significantly by 3.76 ± 0.34 log10 GE/g compared to PID 0. The viral RNA was also detected in the leaves of lettuce plants until PID 28 (Table 1). However, the RNA levels were near or below the infectivity detection limit, and as expected, infectious viral particles from leaves that were positive for SaV viral RNA were not detected. The SaV RNA was not detected in any sample (leaves or roots) of the control plants. Therefore, these results indicated that SaV RNA could be transferred from contaminated roots to leaves of young lettuce plants.

The SaV RNA was not detected in any sample (leaves, roots, or sap) of the control nontreated plants. Therefore, our results showed that SaV RNA could not be transferred from contaminated soil to roots and leaves of mature plants (8 weeks of age). The SaV inoculated onto lettuce, the qRT-PCR detection limit was 2.4 ± 0.26 log10 GE/g of lettuce tissue.

The SaV can be transferred from contaminated soil to roots of young plants (4 weeks of age). The SaV RNA persisted on the roots of lettuce plants for at least PID 28 (Fig. 1). The mean SaV RNA titer detected on roots at 2 h postinoculation (PID 0) was 7.87 ± 0.3 log10 GE/g. In comparison to PID 0, the viral RNA titer decreased significantly by 1.65 ± 0.34 log10 GE/g on PID 2 and then was maintained at similar levels through PID 14. By PID 28, the viral RNA decreased significantly by 3.76 ± 0.34 log10 GE/g as compared to PID 0. The viral RNA was also detected in the leaves of lettuce plants until PID 28 (Table 1). However, the RNA levels were near or below the infectivity detection limit, and as expected, infectious viral particles from leaves that were positive for SaV viral RNA were not detected. The SaV RNA was not detected in any sample (leaves or roots) of the control plants. Therefore, these results indicated that SaV RNA could be transferred from contaminated roots to leaves of young lettuce plants.

The SaV can be transferred from contaminated soil to roots and leaves of mature plants (8 weeks of age). The SaV mean titer of 4.08 ± 0.41 log10 GE/g was detected at 2 h postinoculation (PID 0) in the roots of lettuce plants that were inoculated through the soil. Viral RNA was detected in the roots through at least PID 14 without any significant differences in titer (Fig. 2). However, the viral RNA could be detected only in the leaves of three plants (out of nine plants) on PID 0 and PID 2, with mean titers of 2.3 ± 0.19 and 2.99 ± 0.55 log10 GE/g, respectively. To assess whether the virus is transported through the roots into the leaves, the xylem sap exuding from the cut stems from the root side of lettuce plants were also tested for SaV RNA. Our results indicated that SaV RNA could be detected, with various frequencies, throughout the experiment in those saps (Table 2). Overall, the SaV RNA titers in the saps were not significantly different from each other. The SaV RNA was not detected in any sample (leaves, roots, or sap) of the control plants. Therefore, our results showed that SaV RNA could be transferred from contaminated soil to roots and leaves of mature lettuce plants, through the xylem vessels.

The SaV was not transferred from contaminated outer leaves to inner leaves of mature plants (8 weeks of age). The SaV inoculated onto lettuce outer leaves persisted for at least PID 14 as shown by the detection of the viral RNA on those leaves (Fig. 3). Compared to PID 0, the viral RNA titers decreased significantly an

TABLE 1 Mean log_{10} SaV RNA (GE/g) detected in leaves of lettuce plants that were inoculated through their roots

<table>
<thead>
<tr>
<th>PID</th>
<th>No. of positive plants/no. of total replicates</th>
<th>Mean log_{10} of SaV RNA (GE/g) in positive lettuce leaf samples (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4/9</td>
<td>5.20 ± 0.35</td>
</tr>
<tr>
<td>2</td>
<td>6/9</td>
<td>4.10 ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>2/9</td>
<td>3.56 ± 0.26</td>
</tr>
<tr>
<td>7</td>
<td>1/9</td>
<td>3.97</td>
</tr>
<tr>
<td>14</td>
<td>2/9</td>
<td>3.66 ± 0.25</td>
</tr>
<tr>
<td>28b</td>
<td>2/9</td>
<td>2.83 ± 0.29</td>
</tr>
</tbody>
</table>

* The SaV RNA was not detected in a ry of the control nontreated plants.
  a The qRT-PCR detection limit was 2.4 ± 0.26 log_{10} GE/g of lettuce tissue.

TABLE 2 Mean log_{10} SaV RNA (GE/ml) detected in xylem sap of lettuce plants that were inoculated through the soil

<table>
<thead>
<tr>
<th>PID</th>
<th>No. of positive sap/no. of total replicates</th>
<th>Mean log_{10} SaV RNA (GE/ml) of positive xylem sap samples (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4/9</td>
<td>5.47 ± 0.26</td>
</tr>
<tr>
<td>2</td>
<td>3/9</td>
<td>4.83 ± 0.27</td>
</tr>
<tr>
<td>5</td>
<td>3/9</td>
<td>5.10 ± 0.55</td>
</tr>
<tr>
<td>7</td>
<td>3/9</td>
<td>4.79 ± 0.10</td>
</tr>
<tr>
<td>14</td>
<td>1/9</td>
<td>4.78</td>
</tr>
</tbody>
</table>

* The SaV RNA was not detected in sap of the control nontreated plants.
average of 1.2 ± 0.15 log₁₀ GE/g on PID 2 and continued to decrease by about 3.25 ± 0.35 log₁₀ GE/g through PID 14. The mean infectious viral titer detected at 2 h postinoculation was 4.6 ± 0.10 log₁₀ TCID₅₀/g. However, no infectious viruses could be detected on any other PID. The SaV RNA was not detected in any of the inner leaves of infected plants nor was it detected in control plants at any PID. Overall, our results showed that SaV could not be transferred from outer leaves to inner leaves under our experimental conditions, but the viral RNA may persist on contaminated outer leaves until harvesting time.

**Lettuce leaf milky sap affects SaV infectivity and antigenicity.** The infectivity of SaV incubated with milky (latex) sap diluted 1:5 in sterile water was significantly reduced by 2.12 ± 0.45 log₁₀ compared to that of SaV treated with sterile water (Fig. 4A). Diluting the latex sap further (1:10) showed a lesser but significant reduction of SaV infectivity (1.17 ± 0.40 log₁₀). Similarly, boiled sap showed a lesser but significant reduction (0.94 ± 0.23 log₁₀) of SaV infectivity (Fig. 4A). However, latex sap did not affect the viral RNA titer at any treatment (Fig. 4B).

The antigenicity of SaV incubated with latex sap (1:10) was assessed using two polyclonal antibodies against SaV in an Ag-ELISA sandwich assay. Lettuce latex sap significantly reduced the antigenicity of SaV whether it was boiled or not (Fig. 5). By boiling SaV and then mixing it with sap, the antigenicity was reduced by over 85%. Neither the xylem sap nor the aqueous lettuce leaf extracts showed any significant effects on SaV infectivity or antigenicity (data not shown).

**DISCUSSION**

Despite the increased recognition of fresh produce as a vehicle for human viral pathogens, little is known about the transmission route and the persistence of these viruses in leafy greens. We first tested the possible transfer of SaV directly from roots to leaves in young lettuce plants. Since viruses are known to adsorb to clay particles in soil (8), the roots of the plants were washed completely free of soil before treatment with SaV. The presence of viral RNA (which is labile in nature) within the leaves suggests that viral particles were transferred to the leaves. Specifically, the virus was capable of transferring to young leaves within 2 h postinoculation (Table 1). The minimal damage occurring while washing soil from the roots, in addition to removing the soil barrier, might explain the rapid transfer of the virus to the leaves. Damage to the roots is common under field conditions and also during transplantation or cultivation (60). These results support previous studies showing the detection of CaCV and MNV RNA in leaves of young lettuce plants as early as PID 1, with or without root damage (55, 61). Second, since the physiology of young lettuce plants does not necessarily mimic that of mature plants, we assessed the transfer of SaV from soil to roots and then leaves of mature plants grown in soil pots without any physical damage to their roots. Although SaV RNA was detected only in the leaves until PID 2, the virus...
RNA was detected in the roots and the xylem sap until PID 14 (Table 2). This result suggests that virus can be transferred within the xylem vessels and that the virus is capable of transferring to the leaves of mature plants grown in soil, with intact roots. Similarly, another study showed that the RNA of CaCV inoculated into the soil of lettuce plants was detected in the leaves and vascular liquid until PID 2 (55). Using fluorescent microspheres (ranging from 1 to 10 μm) applied onto the soil of mature lettuce plants, a previous study found that these microspheres can be taken up as early as PID 1 (50). In addition, studies using different food crops have shown that viruses such as hepatitis A (HAV) and bacteriophage f2 can be taken up by roots of mature plants, such as green onions, corn, and beans (9, 60). Collectively, our results expand on previous studies and show that an enteric calicivirus, porcine SaV, used as an NoV surrogate is capable of transferring through the xylem vessels to contaminate the internal tissues of young and mature lettuce plants.

Another possible route of SaV internalization is through the natural opening of the leaves, the stomata. In the past, stomata were recognized as passive points of entry for plant-pathogenic bacteria. However, recent evidence suggests that they are controlled by certain microbe-associated signals (54, 66). Studies performed on cut leaves of spinach and lettuce using the food-borne pathogenic bacteria *Escherichia coli* O157:H7 have shown that the bacteria can colonize the inside of the stomatal cavity (46, 48). Other investigators showed that the food-borne pathogen *Listeria monocytogenes* can penetrate through plant stomata into the intercellular spaces of cut *Arabidopsis thaliana* leaves (38). Murine NoV and viruses like particles of human NoV have been shown to preferentially accumulate inside stomata of cut lettuce leaves (15, 62). However, none of these investigators evaluated the entry and systemic spread of these pathogens through the stomata using actively growing plants. In our study, SaV RNA was not detected in any inner leaves of lettuce plants whose outer leaves were contaminated with the virus. This result is consistent with those of plant-pathogenic viruses which showed that, unlike plant bacteria, plant viruses were unable to infect plants by entry through the stomata (4). Therefore, our results showed that, under the conditions tested, SaV contamination of outer intact leaves does not spread systematically to other internal leaves.

The viral RNA of SaV introduced directly to the roots of lettuce seedlings persisted on the roots of growing lettuce for at least 1 month. Infectivity of the viral particles on roots was not monitored since the roots are not usually an edible part of the vegetable. However, the viral RNA titers showed significant reduction by PID 28 (Fig. 1). This result suggests that with time, viral particles and the genomic RNA were damaged or lost. The initial decrease in SaV RNA titer at PID 2 in roots of lettuce seedlings could also be the result of transferring more viruses to the leaves and/or reflecting the loss of viral particles adsorbed to soil after planting the seedlings in the soil pot. Viruses associated with the roots could also be exposed to degrading substances found in the soil. For example, plant roots continuously secrete an enormous range of compounds into the surrounding soil, including ions, enzymes, mucilage (polysaccharides), phenolics, and an array of secondary metabolites (2). Some of these compounds function in defense against microbial pathogens (3), which could explain the further decrease in SaV RNA titers. When SaV was introduced through the soil, containing mature plants, only a fraction (~4 log10 GE/g) of the initial inoculum (~10 log10 GE/g) was associated with the roots. This result showed that soils act as a barrier by adsorbing the virus and thereby reducing the viral contamination of roots. Overall, our results suggest that SaV RNA associated with lettuce roots preharvest persisted until harvest.

In order for enteric viruses to be transmitted by food and cause disease, the virus must survive and persist until the food is ingested by susceptible hosts (30). Plant leaves have unique physicochemical properties and are exposed to environmental conditions to which human and animal pathogens may not be adapted to survive (5). It is therefore important to examine the preharvest persistence of infectious enteric viruses on leafy greens up to harvesting. We assessed the persistence of SaV in leaves of growing lettuce seedlings and on outer leaves of mature lettuce plants. Although the SaV RNA persisted on the outer leaves of mature lettuce until PID 14 (market-ready lettuce), infectious virus could not be detected at PID 2 or thereafter. Since the viral RNA titers followed a decreasing trend (Fig. 3), it is possible that certain damaging factors affected the virus integrity and RNA genome on the surface of leaves. Many factors can affect virus infectivity on food surfaces, including temperature, degree of drying, and the type of food surface (22). Most of the studies dealing with the survival of enteric viruses on leaf surfaces were performed on cut leaves under laboratory conditions. For example, infectious FCV on cut lettuce leaf surfaces persisted for 3 days at room temperature versus 7 days at 4°C (36). It is possible that lower temperatures may result in better survival of viruses as a result of slowing down the action of degrading compounds found on the leaf surface. Many plant leaves continuously secrete substances in small quantities that form the normal leachates on the leaf surface (6). These leachates might act as antimicrobials; for example, the leaf surface leachates of apple and tobacco have been shown to inhibit fungal pathogens (6). A previous study comparing the persistence of infectious

![FIG 5 Ag-ELISA showing the effect of lettuce leaf latex sap on SaV antigenicity following 16 h of incubation at room temperature. Latex sap was extracted from plants that were 10 weeks of age. Boiling was performed at 100°C for 5 min. Results are based on three independent experiments with triplicate samples. Data are expressed as means ± SE. Means with different letters differ significantly (P < 0.05).](image-url)
MNV on cut lettuce leaf surfaces to nonbiological surfaces showed that the virus was inactivated more rapidly on lettuce (17). The latter study suggested a potential role for lettuce leaf cells in viral inactivation. This is partially supported by our results showing that infectious SaV incubated in test tubes under the same condition as lettuce plants in the growth chamber persisted until at least day 6 (data not shown), suggesting faster deactivation of infectious SaV on lettuce leaf surfaces. Collectively, our results show that, under the condition tested, SaV infectious particles have limited survival times on the surface of lettuce leaves.

In the leaves of lettuce seedlings that were directly contaminated through the roots, SaV RNA was detected throughout the 1-month period (Table 1). However, in the case of mature plants with indirect contamination of roots, SaV RNA was detected only until PID 2, although it was occasionally detected in the xylem sap until PID 14. Viral infectivity was below our detection limit. Similar to our results, a previous study reported that when the soils or nutrient solution of 3-week-old lettuce plants were contaminated with MNV, the viral RNA was detected for at least PID 5 in the leaves. However, infectious viruses could not be detected under either condition when using a titer (5 × 10⁵ PFU/ml) similar to our viral inoculum (10⁵ TCID₅₀/ml) and in spite of replacing the inoculum every day for 5 days (61). However, only by using an extremely high-inoculum titer (5 × 10⁶ PFU/ml) for 24 h was infectious MNV detected daily (<10³ PFU/ml) until PID 5 when lettuce was grown in nutrient solutions but rarely when lettuce plants were grown in soil (61). Since the genomic RNA of these enteric viruses can often be detected inside the leaves, but infectious virus is less often detected, it is possible that the stability of the virus particles within the growing plant tissues is affected. A previous study using bacteriophage Φ2 as a model to study viral uptake in corn and beans has shown that the virus can be taken up into different parts of the plant; however, the viral infectivity varied depending on the plant tissue to which it translocated (60). Several compounds within the different parts of plants may inactive internalized viruses (30). For example, phenolic substances which are found in many fruits and vegetables significantly reduced the infectivity of poliovirus (27–29). In our study, aqueous extracts of lettuce leaves incubated with SaV did not significantly reduce SaV infectivity or antigenicity (data not shown).

Lettuce leaves secrete and accumulate secondary metabolites, forming the milky sap (latex) inside continuous tubes (laticifers) which are closely associated with the vascular tissues (12, 43, 49). Incubating the latex sap with SaV significantly reduced the infectivity but not the viral RNA titers (Fig. 4), suggesting that the latex sap affected the viral capsids but not the genomes. Ag-ELISA has been used previously to show the negative effects of disinfectants on virus antigenicity (33, 34). Using Ag-ELISA, we showed that the latex sap affected the antigenicity of SaV (Fig. 5). Since boiling latex sap still impacted, but to a lesser extent, the infectivity of SaV, it is possible that latex sap induced damage to the virus capsid and/or physically coated the particles. However, previous studies analyzing the metabolic and proteomic profiles of lettuce milky sap revealed the presence of numerous viral and defense-related proteins (12) as well as secondary metabolites that are antimicrobial, insect antifeedants, and antifungals (49). Similar to our results, latex isolated from other plants, such as the fig fruit and the Jatropha species, were also reported to have antiviral activities against herpes simplex virus 1, echovirus type 11, and adenovirus and tobacco mosaic virus (31, 32). On the other hand, lettuce xylem sap did not have any significant effect on SaV infectivity and antigenicity (data not shown). Xylem vessels function mainly in the transport of mineral-containing water and nutrients absorbed from the soil to the aerial tissues. The xylem sap also contains low concentrations of proteins, amino acids, and sugars (7, 52). Although some of these proteins function in defense reactions (7), we did not detect any antiviral activities against SaV from lettuce xylem sap, which might be due in part to the low concentration of these compounds in the xylem sap. Overall, our results indicated the presence of compounds in the lettuce leaf milky sap that reduced SaV infectivity, thus affecting the detection of infectious viruses within the lettuce leaves.

In conclusion, we have shown that porcine SaV, used as a human NoV surrogate, was not transferred systemically from contaminated outer leaves to inner leaves under the conditions tested. Therefore, removal of outer lettuce leaves directly following a contamination event with enteric viruses might minimize risk to consumers. In addition, we have shown that SaV is capable of transferring from roots to leaves of young and mature lettuce plants through the xylem vessels. Because the viral RNA persisted, with a decreasing trend in the roots, and the stability of infectious SaV is negatively affected by certain compounds found in the lettuce leaf sap, one-time contamination events that happen early in young lettuce seedlings or mature plants weeks prior to harvesting might be diminished by the harvesting time. However, because contamination events can happen over long time periods and the NoV infectious dose is very low, internalized viruses whose infectivity are below detection limits of current assay methods are still of particular concern, since these are unlikely to be removed by washing or current sanitization methods that target the surface of vegetables. Therefore, more rigorous preharvest controls to reduce initial contact of NoV with vegetables are necessary.

ACKNOWLEDGMENTS

This project was supported by The National Research Initiative grant 2007-02085, Agriculture and Food Research Initiative Competitive grant 2011-67017-30067, and grant 2011-68003-30395 from the USDA National Institute of Food and Agriculture. Salaries and research support were provided by state and federal funds provided to the Ohio Agricultural Research and Development Center (OARDC), The Ohio State University.

We thank Zufan Worku for her help in RNA extraction and Melanie Lewis-Ivey and Sally Miller for facilitating the use of the growth chambers and the greenhouse facility. We also thank Ismat I. Kassem, Tomoiichiro Oka, and Melanie Lewis-Ivey for their critical review of the manuscript.

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September 2012 Volume 78 Number 17 aem.asm.org 6277


